EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF *AMBLYOMMA AMERICANUM* TICK SALIVA PROTEIN, BP6CONTIG8.

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Expression and Functional Characterization of Amblyomma americanum Tick Saliva Protein, BP6contig8. (May 2014)

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In an attempt to feed on their hosts, ticks secrete saliva proteins to disrupt host defense mechanisms to tick feeding such as blood coagulation and inflammation. To discover tick proteins that are injected into the vertebrate host during tick feeding, a 24h *Amblyomma americanum* phage display expression library was immuno-screened using antibodies to 24h A. americanum tick saliva proteins. While there are some tick saliva proteins that have known functions, there are many that have yet to be characterized. The goal of this undergraduate thesis research is to gain insight into the function(s) of BP6contig8 (BP6C8) in tick feeding physiology. Data in this research suggest that BP6C8 is likely associated tick feeding events during the first five days of tick feeding as revealed by qualitative RT-PCR analysis of 24-120h fed tick cDNA. The major goal of this thesis research has been achieved: recombinant (r) BP6C8 was expressed in insect cells. The remaining experiments will be to optimize large scale production of rBP6C8 and gauging insight into role(s) of this protein in tick feeding physiology.

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I would like to thank my research advisor Dr. Albert Mulenga of Texas A&M University for his guidance and assistance through the process of amplification and determining functional characteristics of the *Amblyomma americanum* saliva protein, BP6contig8. I would also like to thank the graduate students in Dr. Mulenga's lab for always being available to help out and answering all of my numerous questions throughout the year. I would also like to thank my fiancé, Kenton Farrow, for always being supportive with my work and for helping to push me through the more difficult times during my research.

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CHAPTER I

INTRODUCTION

The tick salivary gland (SG) is among the most important tick organs that play important roles in tick feeding physiology and implicitly tick borne disease agent transmission. In addition to its roles in tick feeding physiology, the tick SG plays other important functions such as osmoregulation, which allows the tick to regulate the amount of water it obtains during periods without blood meals (Bowman et. al. 2004). The tick is able to secrete a fluid with a high salt concentration through the mouthparts to pick up water vapor in the surrounding air, which is then used as hydration. This process is performed when the tick is off the host. While the tick is feeding, more fluids (i.e. blood) are obtained so excess fluids need to be excreted through the salivary gland (Bowman et al. 2004).

Each development stage (larva, nymph, and adult) requires one blood meal before transitioning to the subsequent development stage. Within 5-30 minutes of inserting the hypostome into host skin, hard ticks secrete an amorphous adhesive substance called cement to anchor itself onto host skin. Soft ticks mainly rely on deep penetration and not cement due to the acute amount of time they feed (Sauer et al. 1997). Ticks are pool feeders. They accomplish feeding by lacerating host tissue and blood vessels and ingesting blood that bleeds into the feeding lesion. This feeding style provokes host tissue repair responses such as vasoconstriction, inflammation, platelet aggregation, blood clotting, all of which are aimed at stopping further blood loss. To avoid being noticed by the host, ticks secrete saliva proteins to suppress inflammatory response by the host. The saliva contains proteins that aid in continual blood flow due to anticoagulation factors as stated previously. Once the tick is fully engorged, it will then spontaneously fall off the host. The most important process that occurs and is of most concern is the uptake of pathogens as the tick feeds on a host that carries such pathogen. The majority of pathogens that are acquired by the tick are taken to the midgut and travel to the SG (Sauer et al. 1997). The only pathogen that directly affects the SG is protozoa involved in Babesia spp. and Theileria (Sauer et al.1997).

Ticks and tick borne diseases have a global veterinary and public health impact (Sonennshine 1993). Some of those diseases include Lyme disease, Babesiosis, Tularemia and Rocky Mountain Spotted Fever to name a few. Control and/or management of tick and tick borne disease impact on public and veterinary health is dependent on the use of chemical acaricides. While chemical acaricides are effective in the short-term, they do not offer a permanent solution because of serious limitations such as ticks becoming resistant and acaricide pollution of the environment and food chain contamination (Nodari et al. 2012). To solve this problem, there are attempts to develop vaccines to prevent the transmission of harmful pathogens, such as Borrelia burgdorferi, the causative agent of Lyme disease (Bhattacharya et al. 2011). Vaccines against tick feeding have been advocated as an alternative to acaricide use. The argument is that by impacting tick physiology, and its ability to acquire, maintain and transmit tick borne disease agents, we can interfere with the spread of tick borne diseases. The starting point towards development of tick vaccine is discovery of effective target anti-tick vaccine antigens. The purpose of my research is to produce the recombinant protein of one of *Amblyomma americanum* tick saliva protein, BP6contig8 that was discovered in Dr. Mulenga’s laboratory.

CHAPTER II

MATERIALS AND METHODS

BP6contig8 primer design and amplification

The *A. americanum* ticks used in this research were purchased from labs at Texas A&M University and/or Oklahoma State University. Various time-points were used with the ticks to determine when the BP6contig8 and multiple proteins were secreted; all tissues such as salivary gland (SG), midgut (MG), ovary (OV), including whole tick were obtained from unfed ticks as well as 24h, 48h, 72h, 96h, and 120h fed ticks. Procedures to extract RNA to make cDNA were followed (Chalaire et. al, 2011). Sequences were obtained through published documentation and primers (Table 1) were designed with the use of MacVector software and ordered through the IDT website.

Primer	Primer sequence
BP6contig8 Forward ORF	5'-ATGGTGGCTTTCAAGGCGGCCCTCCTC-3'
BP6contig8 Forward Conserved	5'-CTGCCAGTCGGCGGCCGAGCCCCCGC-3'
BP6contig8 Reverse Conserved	5'-GGTAGTCCATGCAGGCCTGGTACAGC-3'

Table 1. Sequences of primers ordered for initial PCR protocol for amplification of BP6contig8. The forward primer contains the start codon and the reverse conserved primer was obtained by removing the stop codon and going from the antisense direction.

RT-PCR: Expression analysis of whole tick *A. americanum* tissue at different feeding stages

To determine the level of expression of BP6contig8, a RT-PCR (reverse transcriptase polymerase chain reaction) was performed with whole tick cDNA samples from *A. americanum* for unfed, 24hr, 48hr, 72hr, 96hr and 120hr fed ticks. The 10µL reaction was composed of 5µL 2x Go Taq, 1µL of both the BP6c8 gene specific primers (forward ORF and reverse conserved), 1μL of cDNA template, and 2μL of nuclease free water. The same reaction was created for actin, but instead the actin primers were used in place of the BP6c8 primers. Samples were ran in the thermal cycler with conditions consisting of denaturation at 94^{o}C for 3 minutes, followed by 35 cycles of amplification at 94^{o}C for 30 seconds, 58^{o}C for 30 seconds and 72^{o}C for 1 minute, with an extension period at 72^{o}C for 5 minutes. The RT-PCR samples were resolved on a 2% agarose gel containing 1μg ethidium bromide in Tris-acetate-EDTA (TAE) buffer, against a 100base pair ladder (Promega) for 20 minutes at 100V (Chalaire et al. 2011).

Amplifying mature protein coding cDNA

A PCR was conducted (10μL) using whole tick cDNA from unfed, 24h of feeding, 48h, 72h, 96h, and 120h of feeding. Components of the 10μL reaction included 5μL My Taq (BioLine, Taunton, MA), 1μL each of BP6contig8 Open Reading Frame (ORF) forward primer and conserved reverse primer, 1μL of the cDNA for each time point and 2μL of nuclease free water. The six reaction tubes were mixed and inserted into a thermal cycler with conditions consisting of denaturation at 94^{o}C for 3 minutes, followed by 35 cycles of amplification at 94^{o}C for 30 seconds, 58^{o}C for 30 seconds and 72^{o}C for 1 minute, with an extension period at 72^{o}C for 5 minutes. To verify the PCR reaction was successful, products were electrophoresed at 100V for 20 minutes on a 2% agarose gel containing 1μg ethidium bromide in Tris-acetate-EDTA (TAE) buffer, against a 1kb ladder (Promega) (Chalaire et al. 2011). The desired bands from the gel were extracted using a sterile blade and inserted into 1.5mL tubes for gene clean procedures using Promega Wizard SV Gel and PCR Clean-Up System (Madison, WI). The tubes were weighed (0.41g and 0.48g) to determine the amount of solubilizing solution needed to be added for each tube. The 0.41g tube received 1025μL of solubilizing solution while the 0.48g tube received 1200μL. Both tubes were added to a heat block at 50^{o}C and mixed every 5 minutes.

Once both tubes were completely solubilized, 750µL of solution from each tube was added to a column and centrifuged for 1 minute at max speed (this step was repeated until all of the solutions from both tubes were centrifuged). To wash the genetic material caught in the column, 700µL of ethanol (EtOH) was added to each tube and centrifuged for 1 minute. Both tubes were then centrifuged for an additional 3 minutes to ensure all remnants of EtOH eluted out of the column. The columns were transferred to new 1.7mL tubes and centrifuged for 5 minutes. The genetic material was washed through the column upon the addition of 20µL of autoclaved water and incubated at room temperature for 10 minutes. Both tubes were condensed in the SpeedVac for 20 minutes.

Cloning into pGEMT

To construct the rBP6contig8 plasmid a 10µL solution was made with 3.5µL of BP6contig8, 1µL T4 DNA ligase enzyme, 5µL 2x ligase buffer and 0.5µL pGEMT vector (Promega). To determine no EtOH contamination, a 6µL solution was made to run through gel electrophoresis for 20 minutes (1µL of 6x loading buffer, 1µL of BP6contig8 gene clean solution, 4µL water). The gel was visualized under UV light and verified no EtOH contamination due to the presence of bands.

Transformation of the rBP6contig8 gene into DH5α bacteria was performed with 5µL of the BP6contig8 ligation solution and thawed DH5α bacteria from the -80C freezer. The mixed solution was put on ice for 20 minutes then performed heat shocking at 42^{o}C for 30 seconds. The solution was then put on ice for an additional 2 minutes to close the pores and 250µL of SOC media was added to the solution before being put in a shaker for 1 hour at 37^{o}C. One hundred microliters of the media was plated on a 75µg/mL ampicillin infused agar plate to inhibit unwanted bacterial growth and only allow the desired DH5α bacteria to grow, which incubated overnight at 37°C. Once colonies formed, they were numbered, spotted on a masterplate and used for insert checks, which later confirm if the desired bacteria with the gene grew. Insert check solutions for each colony (8) consisted of 5μL Go Taq Green Master Mix (Promega), 2μL BP6contig8 ORF forward primer, 2μL BP6contig8 onserved reverse primer, and 1μL autoclaved water. The conditions for the thermal cycler consisted of denaturation at 94°C for 3 minutes followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute, then extension period at 72°C for 5 minutes. Solutions were run through gel electrophoresis to determine which colonies from the initial plate contained the desired gene. All colonies, except #4, contained the gene and two samples from colonies 2, 6, and 7 (6 total) were used for further amplification. A 7mL solution of SOB infused with ampicillin was used for each of the six samples and put into 15mL tubes. Two samples were taken from each of the three colonies and added into the indicated tubes to be put in the shaker at 37°C overnight.

Miniprep procedures from Wizard Plus SV Minipreps DNA purification System (Promega) were employed for purification of the recombinant plasmid. Once purified, the plasmids were quantified using U640 spectrometer (Beckman, Brea CA). Plasmids containing a concentration reading of more than 400μg/mL were used in the Big Dye sequencing reaction (Applied Biosystems, Grand Island, NY), consisting of 2μL of Big Dye, 1μL of the T7 forward or SP6 reverse primers, 1-1.5μL of the plasmid (depending on the concentration from quantification) and 1.5-2μL of nuclease free water. Following sequencing PCR reaction, samples were purified using spin columns and dried using SpeedVac. Samples were labeled and sent for in-house sequencing at the Noh lab on the Texas A&M University campus. Sequences were analyzed and verified with the use of MacVector and Blast website.

Designing rBP6contig8 expression plasmid

The mature rBP6contig8 protein was constructed using primers designed with added restriction enzymes to facilitate unidirectional cloning into *Spodoptera frugiperda* (Sf) insect cell expression plasmid, pIB/V5 (Invitrogen). The forward primer included the Kozak sequence (GTA ATG G) and sequences for EcoR1 restriction enzyme site (New England Biolabs, Ipswich, MA, USA). The reverse primer excluded the stop codon and also contained the sequence for Not1 restriction enzyme (New England Biolabs, Ipswich, MA, USA). Procedures and kit systems for PCR, Gene Clean, DH5α transformation and Minipreps were repeated for use in pGEMT vector (Promega) according to the manufacturer's instructions.

BP6C8 cDNA insert release from pGEMT vector

Extraction of the mature rBP6contig8 protein was possible through the use of sequential enzymatic digestion with forward Kpn1 and reverse BamH1 restriction enzymes. First, a 100µL reaction containing 20µL of the miniprep solution, 10µL of 10x buffer 3, 2µL of BamH1 restriction enzyme, 1µL of 100x BSA, and 67µL of autoclaved water was made and incubated overnight at 37^{o}C. Second, a PCR Purification (Promega) was run per manufacturer's instructions on the 100µL digested solution. Lastly, the second digestion with Kpn1 restriction enzyme was made to completely release the mature BP6contig8 protein from the pGEMT vector by making a 100µL solution of 40µL of DNA from the purification, 10µL of 10x buffer 1, 3µL of Kpn1 restriction enzyme, 1µL of 100x BSA and 46µL of autoclaved water. The solution was incubated at 37^{o}C overnight. Twenty microliters of the double digested solution was ran on a 2% agarose gel containing 1µg/mL ethidium bromide for 25 minutes at 100V. Gene fragments were extracted using a sterile blade and a gene clean was ran as previously stated.

Insertion into pIB/V5 His by T4 Ligation

BP6contig8 was inserted into digested pIB/V5 His expression plasmid that was previously digested with Kpn1 and BamH1 restriction enzyme. A 10µL reaction containing 7.5µL of the BP6C8 cDNA, 1µL of T4 DNA ligase enzyme, 1µL of T4 DNA ligase buffer and 0.5µL of the digested pIB/V5 vector was made and incubated at 16^{o}C overnight. Following the ligation, the inserted vector was transformed into DH5α bacteria cells using the heat shock method as previously stated and grew overnight at 37^{o}C on an ampicillin infused LB agar plate. To verify insertion, insert check PCR was conducted. In this reaction, a toothpick of pipette tip was touched onto the bacteria colony and then washed into a 10µL reaction that included 5µL of 2x My Taq, 1µL each of the forward and reverse pIB/V5 vector specific OpIE primers and 3µL of nuclease free water. The thermal cycler conditions included 94^{o}C denaturation for 3 minutes, 35 cycles of 94^{o}C for 30 seconds, annealing at 50^{o}C for 30 seconds, 72^{o}C for 1 minute, and then 72^{o}C for 5 minutes to cool. In addition to the PCR insert check reaction, positive colonies were then spotted on another ampicillin infused LB agar plate. The PCR reactions were ran on a 2% agarose gel for 20 minutes and visualized using UV light. Miniprep procedures were done as previously stated using the Promega kit was used after ligation. A 6µL Big Dye sequencing reaction was created using 0.5µL tubes containing 2µL of Big Dye, 1µL of OpIE forward and reverse primers, 1µL of two miniprep solutions and 2µL of nuclease free water. Samples were put in the thermal cycler with the following conditions: 96^{o}C denaturation for 2 minutes, and 35 cycles of 96^{o}C for 30 seconds, 50^{o}C annealing for 15 seconds, 60^{o}C for 4 minutes. Steps for per previously stated transformation protocol were followed to amplify the expression plasmid. Sequencing reactions were followed via lab protocol.

Transfecting *Spodoptera frugiperda* (Sf) 21 insect cells

Following verification of sequences within the correct frame of the expression vector, the pIB/V5-BP6C8 plasmid was purified using SNAP Miniprep Kit (Invitrogen, Carlsbad, Ca) to eliminate endotoxins. For transfection into Sf21 insect cells, the pIB/V5-BP6C8 plasmid was mixed with lipofectin, a lipid compound for 30 minutes at room temperature. Following incubation the lipid and pIB/V5-BP6C8 plasmid mixture was added to Sf21 cells for 2 days. Subsequently transfected cells were grown in serum free media (SF900 II). Both spent media and Sf21 cell samples were collected 48hr, 72hr and 96hr to determine which day had the most expression (described below)

Collection of Spent Media

Media samples followed two separate protocols. First, 30µL of media was added to 10µL of 4x loading buffer and heat denatured at 95^{o}C for 5 minutes. Second, 1mL of media was mixed with 4mL of cold acetone and chilled at -20^{o}C for 1 hour. Samples were alloquated into 2mL tubes and centrifuged for 10 minutes at 15,000g's and 4^{o}C. Supernatant was discarded and to allow the remaining acetone to evaporate, the lids of the tubes were open and placed under a hood for 30 minutes. Once dried, 30µL of 1x PBS buffer was added to one sample to resuspend the pellet into solution. The resuspended solution was then used to resuspend other samples of the same collection date to create a concentrated solution. The end concentrated solution was then mixed with 10µL of 4x loading buffer and heat denatured at 95^{o}C for 5 minutes. Insect samples were made ready for western blotting by centrifuging collected samples at 1,500g's for 5 minutes at room temperature. The supernatant was removed and cells were resuspended using 1x sample buffer, a mixture of 25µL of 4x loading buffer and 75µL of 1x PBS buffer. Samples were then heat denatured at 95^{o}C for 5 minutes.

Verification of recombinant (r)BP6C8 Expression through Western Blotting

To verify rBP6C8 expression spent media and insect lysates were subjected western blotting analysis using the antibody to the histidine tag. To prepare samples for an SDS PAGE electrophoresis, 15µL spent media aliquots and insect cell lysate samples were mixed with 5µL 4x loading buffer and heat denatured at 95^{o}C for 5 minutes. Making the 15% SDS PAGE included 2.82mL of 40% Acrylamide, 2.82mL of 1M Tris (pH 8.8), 75µL of 10% SDS, 1/74mL of distilled water, 37.5µL of 10% A.P.S and 4.5µL of TEMED solution and stacking gel. Samples were then added to the SDS PAGE and ran in 1x Tris-Glycine buffer for 2 hours at 125V. The gel was then added to a fixative solution on a rocker overnight to fix the protein to prevent diffusion through the porous gel. Following electrophoresis, were electroblotted onto PVDF membranes using standard protocols.

The PVDF membrane was added faced down to 5% blocking solution (1.25g of dry skim milk and 25mL of PBS with tween) and rocked at 4^{o}C overnight. After discarding the blocking solution, PBS with tween was added to the PBDF membrane. Another treatment of 5% blocking solution including 2µL of C-terminus Ab was added to the membrane and rocked for 1 hour. Five PBS with tween washes for 5 minutes each were performed while the membrane was on the rocker; the last wash was not discarded to allow the membrane to stay moist. The PBDF membrane was developed on a 8’11” film by exposure to X-rays.

CHAPTER III

RESULTS

BPC8 mRNA is expressed in unfed and fed ticks through five days of feeding

Figure 1 summarizes BP6C8 expression in unfed ticks and ticks that fed for 24-120hr. Analysis shows the protein is continuously secreted throughout tick feeding and suggests it may have an important role in the process. This called for further investigation into the role BP6C8 plays in tick physiology.

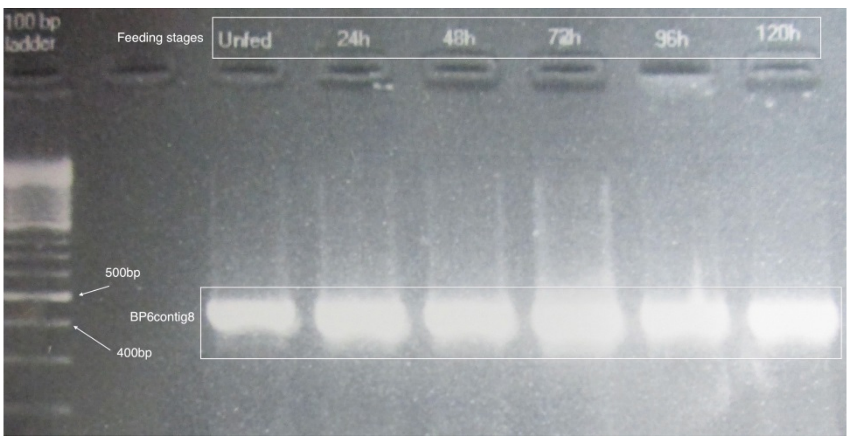

Figure 1. PCR reaction of BP6contig8 with forward ORF and reverse conserved primers. The gel shows expression at the desired size (~430bp) at all time points.

After completion of a gene clean using Promega Wizard SV Gel and PCR Clean-Up System the solution was used for ligation into the pGEMT vector for further amplification.

Qualitative RT-PCR

PCR in Figure 1 show that the BP6C8 mRNA is expressed in the *A. americanum* tick in both unfed and 1-5 day fed whole tick tissues. These results suggest that BP6C8 protein could be associated with tick physiology events off the host and during the parasitic phase when ticks are feeding on animals.

Cloning and DNA sequencing

The 10µL PCR ligation reaction into pGEMT was successful, as seen in Figure 2.

Figure 2. Gene clean of pGEMT ligation reaction

Successful transformation into DH5α cells was apparent from 8 positive colonies on the ampicillin enriched plate and insert checks of those colonies confirmed the presence of BP6contig8. Gene specific primers were used for the PCR insert check reaction, which determines whether or not BP6C8 is present in the colonies sampled. Because all colonies showed bands at the desired BP6C8 400bp size, only colonies 2, 6, and 7 were used for the miniprep procedure, taking 2 samples from each colony to be processed. After extracting the plasmid from the DH5α cells, verification of no ethanol contamination was observed through electrophoresis of miniprep solution.

Quantification of colonies 2A, 6B, and 7A were conducted using a UV Spectrometer (A and B labels indicate sample 1 and sample 2 respectively). Colony 2A had a quantification value between 400-600μg/mL while colonies 6B and 7A were slightly below the desired concentration of 400μg/mL. More of the miniprep samples for colonies 6B and 7A were used for the Big Dye sequencing reaction to compensate for the lower concentration. Capillary sequencing steps were followed to confirm BP6C8 was in frame, with later verification from the sequencing lab confirming it was in frame.

Primers designed for insect cell transfection were used to repeat the process of PCR amplification, insertion into pGEMT vector, and amplification then release from DH5α cells in hopes of inserting BP6C8 into the desired insect cells.

Release from Vector

With the use of Kpn1 and BamH1 restriction enzymes, BP6C8 was released from the pGEMT vector. Removal of BP6C8 from the agarose gel was successfully accomplished through the use of Promega Wizard SV Gel and PCR Clean-Up kit. Though the release showed a faint band for the BP6C8 gene, ligation into pIB expression vector was successful as seen in Figure 3.

Insertion into pIB/V5 His by T4 Ligation

Ligation of BP6C8 into digested pIB/V5 His expression vector was successfully accomplished as seen in Figure 3.

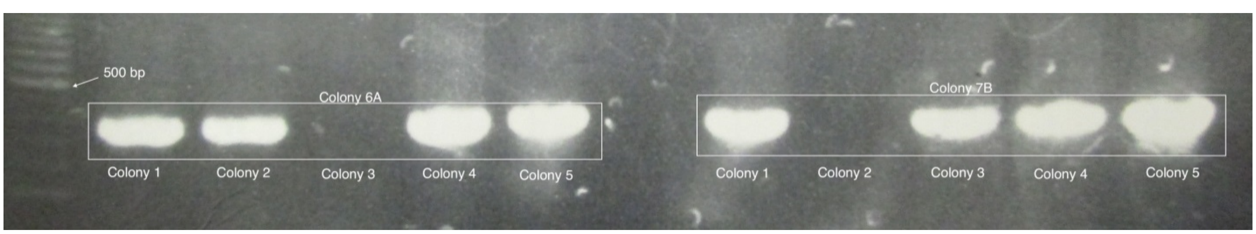

Figure 3. pIB/V5 His expression vector ligation reaction.

All but two colonies were positive for presence of pIB/V5. Transformation into DH5α bacteria cells was accomplished using the previously stated protocol. Miniprep purification protocols were followed to extract and purify the expression plasmid from DH5α. A capillary sequencing reaction followed to determine if expression plasmid was in frame, with later results from the lab indicating it was in frame.

Purification of Expression Plasmid

A SNAP Miniprep kit was used to highly purify the expression plasmid for insect cell transfection. Because the insect cells used are highly sensitive to contamination, the expression plasmid must be purified to a higher degree to ensure no extraneous materials other than BP6C8 and pIB/V5 are present. Quantification results were determined to be sufficient enough with values for the miniprep samples being between the desired 400-600µg/mL concentrations.

Transfecting *Spodoptera frugiperda* (Sf) 21 insect cells

Before the expression plasmid could be transfected, the *Spodoptera frugiperda* (Sf21) insect cells were successfully grown to confluence while contamination was prevented with the use of sterile procedures. To allow the Sf21 insect cells to take up the expression plasmid, lipids were adhered to BP6C8 as markers. The Sf21 cells recognize the lipid and endocytose the expression plasmid and grow/multiply, amplifying BP6C8.

Collection of Spent Media

Both media and insect cell samples were collected at day 2, 3 and 4 of growth to determine the optimal expression time. Insect cells and media were maintained throughout the collection process to ensure no degeneration or contamination throughout the various prepatory protocols.

Verification of Expression through Western Blotting

The development of the Western Blot procedure shown in Figure 4 indicates day 3 has the highest level of expression. Confluent Sf21 cells in T25 flasks were collected after 3 days of developing and the process was repeated along with sampling to create media to be used for future functional assays.

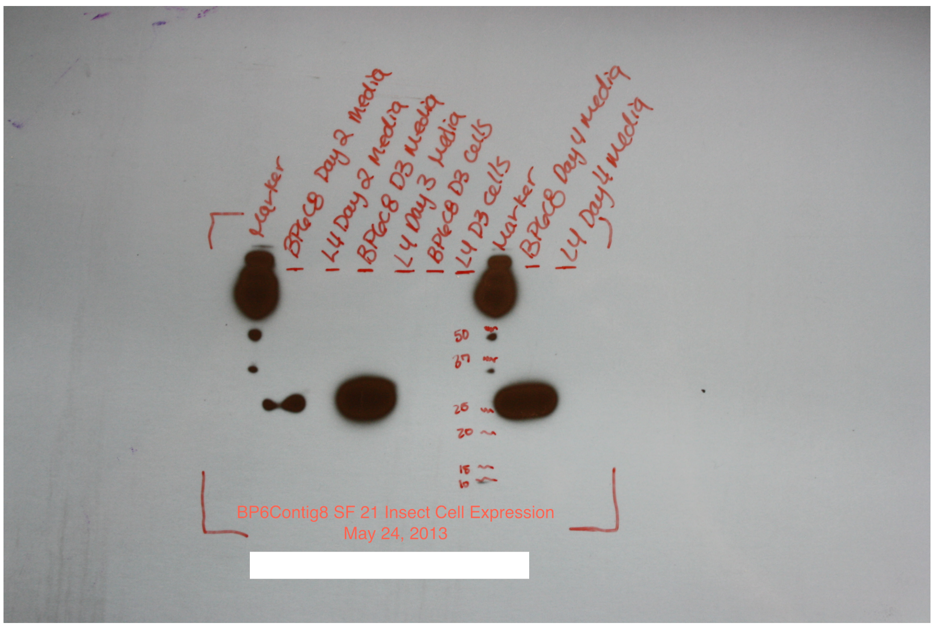

Figure 4. Western Blot development showing BP6C8 gene expression at various days of sampling against a ladder marker.

CONCLUSION

Through the discovery of the tick saliva protein BP6contig8 and amplification through PCR, transformation and transfection, we have determined that the protein is secreted by the tick and is able to be expressed in insect cells. The expression in Sf21 insect cells will allow further research into the functional characterization or role that it plays in the tick feeding process. With this knowledge, hopefully we will develop a vaccine to be administered to mammals as a way to prevent the spread of deadly and debilitating tick borne diseases that affect both agriculture and human popmes

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